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ORIGINAL ARTICLES

Bacterial Pathogens in Ixodid Ticks from a Piedmont County in North Carolina: Prevalence of Rickettsial Organisms

Michael P. Smith,[1] Loganathan Ponnusamy,[1] Ju Jiang,[2] Luma Abu Ayyash,[1] Allen L. Richards,[2] and Charles S. Apperson[1]

Abstract

In North Carolina, reported human cases of tick-borne illness, specifically Rocky Mountain spotted fever, have escalated over the past decade. To determine the relative abundance of vectors and to estimate the risk of acquiring a tick-borne illness in peri-residential landscapes, ticks were collected in Chatham County, a typical Piedmont county and, samples of the ticks were tested for infection with selected bacterial pathogens using real-time quantitative polymerase chain reaction assays. Ticks ($n = 3746$) were collected by flagging vegetation at 26 sites from April to July 2006. The predominant questing tick was *Amblyomma americanum* (98.5%) with significantly fewer *Dermacentor variabilis* (1.0%) and *Ixodes scapularis* (0.5%) collected. Spotted fever group (SFG) rickettsiae were detected in 68.2% of 1590 *A. americanum* with 56.4% of the molecular isolates identified as *Rickettsia amblyommii*, an informally named member of the SFG rickettsiae. Comparatively, smaller numbers of *A. americanum* contained *Ehrlichia chaffeensis* (1.8%) and *Borrelia lonestari* (0.4%). Of 15 *I. scapularis* nymphs tested, 6 (40%) were positive for *Borrelia burgdorferi* sensu lato. Seven (19.4%) of 36 adult *D. variabilis* tested positive for *Rickettsia montanensis*, 4 (11.1%) were positive for *R. amblyommii*, and 5 (13.9%) were infected with unidentified species of SFG rickettsiae. The tick population in Chatham County contains a diverse array of microbes, some of which are known or potential pathogens. Highest attack rates would be expected from *A. americanum* ticks, and highest potential risk of infection with a tick-transmitted agent would be to rickettsial organisms, particularly *R. amblyommii*. Accordingly, longitudinal eco-epidemiology investigations are needed to determine the public health importance of *A. americanum* infected with rickettsial organisms.

Key Words: *Amblyomma americanum*—American dog tick—Black-legged tick—*Borrelia* lone star tick—*Dermacentor variabilis*—*Ehrlichia chaffeensis*—*Ixodes scapularis*—Pathogen prevalence—*Rickettsia*—*Rickettsia amblyommii*.

Introduction

TICK-BORNE ILLNESSES (TBIs) are zoonoses involving pathogens transmitted by ixodid ticks that parasitize wildlife, and humans are incidentally infected. In North Carolina, and other areas of the southern United States, TBIs constitute a significant source of illness. In North Carolina, reported cases of Rocky Mountain spotted fever (RMSF) have increased significantly from 78 to 515 cases between 2000 and 2008; 852 cases reported in 2006 represented 42% of all RMSF cases reported to Centers for Disease Control and Prevention (www.epi.state.nc.us/epi/gcdc.html).

Rickettsia rickettsii, the causative agent of RMSF, is transmitted in the eastern United States by *Dermacentor variabilis*, the American dog tick (Parola et al. 2005). Although *D. variabilis* has been recorded from North Carolina (Slaff and Newton 1993), surveys of its geographic distribution and eco-epidemiology studies documenting infection rates in North Carolina are lacking. In response to the escalation of RMSF cases, a prospective eco-epidemiology investigation of ticks and tick-transmitted illnesses was completed in Chatham County in the Piedmont of North Carolina in 2005 (Apperson et al. 2008). From 2000 to 2005, a total of 35 cases of RMSF were reported from Chatham County (www.epi.state.nc.us/epi/

[1]Department of Entomology, North Carolina State University, Raleigh, North Carolina.
[2]Viral and Rickettsial Diseases Department, Naval Medical Research Center, Silver Spring, Maryland.

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14. ABSTRACT

In North Carolina, reported human cases of tick-borne illness, specifically Rocky Mountain spotted fever, have escalated over the past decade. To determine the relative abundance of vectors and to estimate the risk of acquiring a tick-borne illness in peri-residential landscapes, ticks were collected in Chatham County, a typical Piedmont county and, samples of the ticks were tested for infection with selected bacterial pathogens using realtime quantitative polymerase chain reaction assays. Ticks (n = 3746) were collected by flagging vegetation at 26 sites from April to July 2006. The predominant questing tick was Amblyomma americanum (98.5%) with significantly fewer Dermacentor variabilis (1.0%) and Ixodes scapularis (O.SO/o) collected. Spotted fever group (SFG) rickettsiae were detected in 68.2% of 1590 A. americanum with 56.4% of the molecular isolates identified as Rickettsia amblyommii, an informally named member of the SFG rickettsiae. Comparatively, smaller numbers of A. americanum contained Ehrlichia chaffeensis (1.8%) and Borrelia lonestari (0.4%). Of 15 I. scapularis nymphs tested 6 (40%) were positive for Borrelia burgdorferi sensu lato. Seven (19.4%) of 36 adult D. variabilis tested positive for Rickettsia montanensis, 4 (11.1%) were positive for R. amblyommii, and 5 (13.9%) were infected with unidentified species of SFG rickettsiae. The tick population in Chatham County contains a diverse array of microbes, some of which are known or potential pathogens. Highest attack rates would be expected from A. americanum ticks, and highest potential risk of infection with a tick-transmitted agent would be to rickettsial organisms, particularly R. amblyommii. Accordingly, longitudinal eco-epidemiology investigations are needed to determine the public health importance of A. americanum infected with rickettsial organisms.

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gcdc.html). Tick population samples from 32 residential sites were largely comprised of *Amblyomma americanum* (99.6%), while markedly few *D. variabilis* (0.4%) were collected. A sample of the *A. americanum* ticks ($n = 308$) and all *D. variabilis* ($n = 23$) collected were tested by polymerase chain reaction (PCR) for spotted fever group (SFG) rickettsiae. *Rickettsia amblyommii*, an informally named member of the SFG rickettsiae, was the predominant rickettsial organism identified; notably, *R. rickettsii* was not detected. Concurrently, physician-diagnosed cases of TBI from Chatham County were serologically evaluated by an indirect fluorescence antibody assay (IFA). *R. rickettsii* infections could not be confirmed by IFA as the cause of illness for six patients found to have a TBI by Chatham County physicians. When paired sera for the six patients were retested, three of six exhibited fourfold changes in immunoglobulin G titers to *R. amblyommii* antigens but not to *R. rickettsii* antigens. Taken together, the abundance of *A. americanum*, prevalence of *R. amblyommii*, and higher IFA titers to *R. amblyommii* antigens suggested to Apperson et al. (2008) that rickettsioses reported as RMSF could be caused by *R. amblyommii* transmitted through the bite of *A. americanum*.

Cases of human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis* (McQuiston et al. 1999) and transmitted by *A. americanum* (Anderson et al. 1993, Steiert and Gilfoy 2002), are reported less frequently than RMSF. However, the occurrence of cases indicates that some tick populations in North Carolina are infected with the causal agent of this disease. Reported cases of HME in North Carolina have increased only slightly from 28 in 2003 to 35 in 2008. However, the illness appears to be under-reported. In the eco-epidemiology investigation of Apperson et al. (2008), five patients found to have a TBI by Chatham County physicians were confirmed to be HME cases by laboratory serological tests.

Lyme disease cases are reported each year in North Carolina, including Chatham County (www.epi.state.nc.us/epi/gcdc.html). The illness is caused by the spirochete *Borrelia burgdorferi*, which is transmitted by the blacklegged tick *Ixodes scapularis* (Piesman 1989). *B. burgdorferi* circulates zoonotically in North Carolina (Ouellette et al. 1997, Ryan et al. 2000) and other areas of the southeast (Oliver et al. 2003). However, *B. burgdorferi* infection rates in *I. scapularis* populations appear to be low based on past and recent canine serosurveys (Greene et al. 1988, Duncan et al. 2004). In the northeast, *I. scapularis* nymphs are infected by feeding on spirochetemic white-footed mice (Levine et al. 1985). In the southeast, *I. scapularis* are diverted from feeding on mice by lizards (Apperson et al. 1993, Durden et al. 2002). In addition, *I. scapularis* does not appear to readily bite people in the southeast (Felz et al. 1996, Felz and Durden 1999) and the nymphal stage has rarely been collected by flagging vegetation (Diuk-Wasser et al. 2006, Apperson et al. 2008).

Borrelia lonestari, an informally named spirochete related to *B. burgdorferi*, is suspected of causing a Lyme-disease-like illness with an erythema migrans rash, which is now referred to as Southern-tick-associated rash illness (Taft et al. 2005). A patient with a travel history in North Carolina developed an erythema migrans after being bitten by an *A. americanum* tick and, *B. lonestari* DNA was recovered from both the tick and skin rash of the patient (James et al. 2001). White-tailed deer are considered to be the reservoir for *B. lonestari* (Moyer et al. 2006). *B. lonestari* nucleic acids have been identified in *A. americanum* ticks (Taft et al. 2005, Mixson et al. 2006) and white-tailed deer from North Carolina (Moore et al. 2003).

As suburbanization continues in the Piedmont of North Carolina, development of rural woodlands will create patchy habitats that promote the growth of white-tailed deer populations and, consequently, lone star tick populations. Human contact with pathogen-infected ticks, especially *A. americanum*, will likely increase. Estimating the risk of infection from tick-borne pathogens is a critical determinant of developing targeted methods of disrupting zoonotic disease cycles and reducing pathogen transmission to humans.

In this regard, results of the eco-epidemiology investigation of Apperson and coworkers (2008) suggested that in rural landscapes of Chatham County, highest risk of infection to a tick-transmitted agent was to rickettsial organisms transmitted by *A. americanum*. However, only a small proportion of the ticks from sampling sites were examined, and the ticks were pooled before they were tested, so the accuracy of estimated infection rates could be questioned. The present investigation is an extension of previous research (Apperson et al. 2008) conducted in Chatham County. The principal aim was to estimate the prevalence and spatial distribution of ticks infected with selected pathogens by examining a larger number of ticks and by testing the ticks individually. Additionally, the occurrence and relative abundance of tick species in peri-residential landscapes of Chatham County was further documented.

Materials and Methods

Study area

Chatham County is located in the Piedmont region of North Carolina. The county is representative of rural counties in which the majority of the population presently resides outside of incorporated towns. However, through suburbanization, farmlands are being replaced by peri-residential landscapes. The population increased by 22% from 2000 to 2006; in 2006, the county experienced the 12th highest population growth rate among all counties in North Carolina. Agribusiness accounted for 38% of the economy with 27% of the overall land acreage devoted to farming. Additional demographic information was obtained from federal (http://quickfacts.census.gov/qfd/states/37/37037.html), state (www.osbm.state.nc.us/ncosbm/facts_and_figures/socioeconomic_data/population_estimates.shtm), and county (www.chathamnc.org/Index.aspx?page = 25) sources.

Sampling sites ($n = 18$) were located with assistance from the North Carolina Division of Public Health and the North Carolina Tick-borne Infections Council, a citizens' advocacy group for tick-borne disease issues. Additional sites ($n = 8$) were selected while in route to previously arranged collection sites after conversations with local residents about tick populations. Of the 26 sites, the majority of collections ($n = 17$) were made at private residences where the landscape was comprised of lawn, woodland, and woodland–lawn ecotonal areas. However, some collections were made on small acreage farms with pasture and orchard land ($n = 4$), in public forests ($n = 3$), and around houses built in heavily wooded areas but with some lawn space ($n = 2$). Vegetation at sampling sites typically consisted of cool or warm season grass lawns surrounded or interspersed with oak–pine woodlands with an understory of leaf litter and

low growing shrubs. Sampling at each home site was carried out with consent of the resident.

Collection and processing of ticks

Ticks were collected by flagging vegetation at all 26 sites from April to July 2006 (Fig. 1). The flag was constructed from a 2×1.1 m size piece of gold-colored corduroy, similar to drapery material. At each site, vegetation was flagged in 20 m swaths alternating the flag in a crisscross manner. All vegetative ecotypes (lawn, pasture, woodland, and ecotone areas along woodlands) at each site were flagged to ensure that all habitats potentially harboring ticks were sampled. A minimum of 10 swaths were flagged in each distinct habitat present or ecotone between habitats. After all habitats had been sampled, efforts were made at some sites to collect additional ticks until at least 100 ticks at each site were collected and also to increase the likelihood of collecting *D. variabilis* and *I. scapularis* if present. Additional attempts to collect *I. scapularis* adults during the winter of 2006 and 2007 were made at some sites where nymphs were collected.

At the end of each swath, all ticks were removed from each side of the flag and transferred into a 2-oz. plastic snap cap vial (Fisher Scientific, Fair Lawn, NJ). The vial contained a mixture (10:1 by volume) of Plaster of Paris (DAP, Baltimore, MD) and powdered activated carbon (Fisher Scientific), which provided a humid environment with the addition of a few drops of water. A piece of nitrile glove was held in place over the open end of the vial by a snap-on lid. Ticks were transferred with forceps into the vial through a hole in the lid and a small slit in the piece of nitrile. Vials were kept in a shaded area until the survey at each site was completed and were then placed on wet ice and transported to the laboratory within 3–5 h of collection. In the laboratory, for each collection site, ticks were sorted to life stage, sex, and species, and after counting were placed individually into 1.5-mL microcentrifuge tubes (USA Scientific, Ocala, FL) and then stored frozen at −80°C for subsequent extraction of genomic DNA.

Extraction of DNA

Genomic DNA was extracted from individual ticks in a room separate from other molecular work. Because of the large number of *A. americanum* nymphs collected (~3500), a maximum of 50 randomly selected nymphs were processed from each collection site. All adult ticks and *I. scapularis* nymphs collected were processed. Lysis buffer (150 μL; pH 7.5–8.0) composed of 2% (w/v) sodium dodecyl sulfate (Sigma-Aldrich, St. Louis, MO), 1% (w/v) cetyltriethylammonium bromide (Sigma-Aldrich), 1.5 M sodium chloride (Sigma-Aldrich), 100 mM Tris-HCl (Sigma-Aldrich), and 100 mM ethylenediaminetetraacetic acid (Fisher Scientific) was added to each 1.5-mL microcentrifuge tube. Each tick was minced with a sterile #11 scalpel blade inside the microcentrifuge tube, 20 μL of 1% (w/v) protease K was added, and the preparation was incubated in a water bath for 2 h at 56°C.

After incubation, 200 μL of a solution (pH 7.5–8.0) composed of 5.5 M guanidinium thiocyanate (Amresco, Solon, OH) and 20 mM Tris-HCl was added to each microcentrifuge tube, followed by incubation at 70°C for 10 min. After incubation, 1 μL (10 mg/mL) of carrier RNA (polyadenylic acid; Amersham Biosciences, Piscataway, NJ) was added to each tube to enhance DNA yield (Kishore et al. 2006), followed by brief vortexing and addition of 230 μL of cold absolute ethyl alcohol. After vortexing, the solution was transferred to an EconoSpin™ silica membrane spin column with attached lid (Epoch Bio Labs, Missouri City, TX) and centrifuged at 5900 *g* for 60 s. Five hundred microliters of a wash buffer (pH 7.5–8.0),

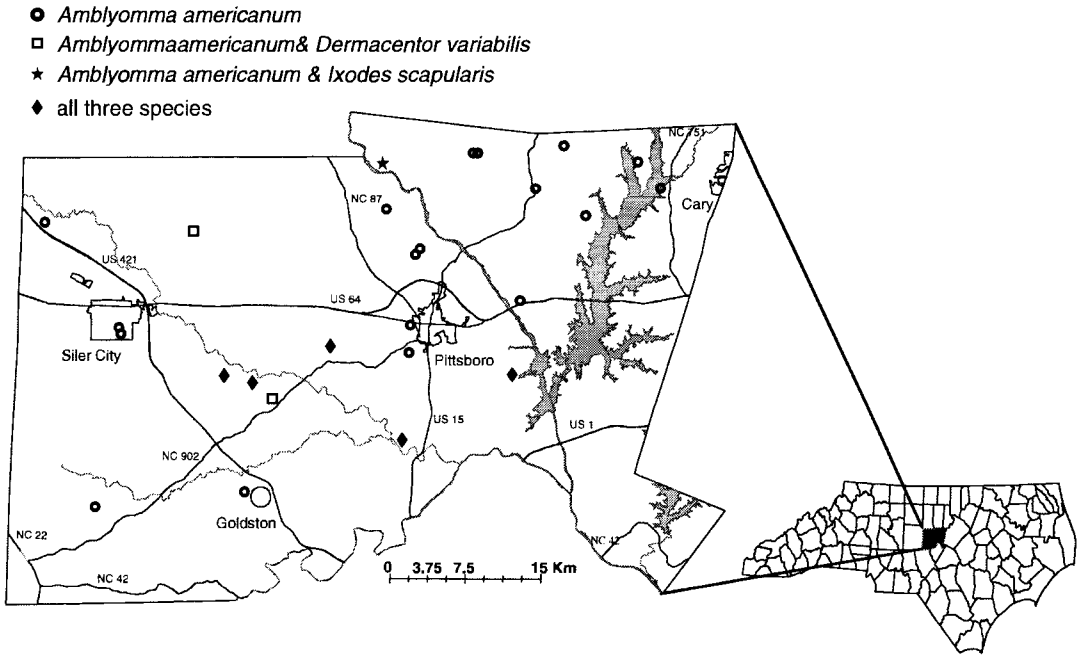

FIG. 1. Species composition of ticks collected at 26 sampling sites in Chatham County, North Carolina, from April to July 2006.

composed of 5.0 M guanidine hydrochloride (Sigma-Aldrich), 20 mM Tris-HCl, and 38% (v/v) ethyl alcohol, was added and each spin column was centrifuged at 5900 *g* for 60 s. Buffer AW2 (500 μL) from the Qiagen DNeasy® Blood and Tissue Kit (Qiagen, Chatsworth, CA) was used in the second wash step, and each column was centrifuged at 15,700 *g* for 3 min to remove alcohol. Genomic DNA was eluted by centrifugation at 5700 *g* for 60 s from the column using DNA-grade water in two separate elusions of 35 and 30 μL each after a 5-min incubation, and then was pooled into a single 1.5-mL microcentrifuge tube. Eluates were stored at −80°C until tested for bacterial pathogens by quantitative PCR (qPCR) as described below.

Detection of putative bacterial pathogens by real-time qPCR

Tick genomic DNA was tested for the presence of selected bacterial pathogen DNA using real-time qPCR assays based on TaqMan and Molecular Beacon probe technology with a SmartCycler amplification and detection system (Cepheid, Sunnyvale, CA). These analyses were completed at the Naval Medical Research Center facilities in Silver Spring, Maryland. All master mix solutions were prepared in an isolated clean room where no molecular samples were allowed. Mixing of final reaction solutions was performed in a laminar flow hood. Probes, primer sets, concentrations, and reaction mixtures are listed in Table 1. Reactions were performed using Platinum qPCR Supermix UDG (Invitrogen, Carlsbad, CA), which contained PCR buffer, 0.2 mM dNTPs, and 0.75 U platinum Taq DNA polymerase, using 2 μL of genomic DNA from individual ticks as template with a total reaction volume of 25 μL. All reactions (with the exception of *E. chaffeensis*, which cycled at 95°C/58°C) were run with the following two-step amplification program: 50°C for 2 min, 95°C denaturation for 2 min, 45 cycles at 95°C for 15 s, and 60°C for 30 s. Reactions were terminated after 45 cycles, and a relative fluorescence cut off value of 30 was set based on software standards, according to the manufacturer's recommendation. Each batch of samples tested included three negative controls each consisting of 2 μL of sterile distilled water and one positive control consisting of 2 μL template DNA from a plasmid-amplified fragment of the appropriate gene target with an estimated copy number of 1000 copies/μL.

A. americanum nymphs and adults were tested individually using the *R. amblyommii*–specific Rambl qPCR assay first (Table 1) because it was anticipated that this agent would be prevalent in lone star ticks (Mixson et al. 2006, Jiang et al. 2009). All *A. americanum* nucleic acid preparations testing negative to the Rambl species-specific assay were then retested with the genus-specific Rick17 qPCR assay (Jiang et al. 2004). All *I. scapularis* and *D. variabilis* ticks were tested individually using the Rick17 qPCR assay. *D. variabilis* ticks testing positive were then assessed for the presence of *R. rickettsii*, *R. amblyommii*, and *Rickettsia montanensis* by Rrick (Jiang et al. 2005c), Rambl, and Rmont (Rozmajzl et al. 2006) qPCR assays, respectively. *I. scapularis* ticks that were Rick17 positive were not tested in species-specific rickettsial qPCR assays. However, all *I. scapularis* were also tested individually for the presence of *B. burgdorferi* sensu lato using a Bburg qPCR assay.

E. chaffeensis and *B. lonestari* infection rates were expected to be less than 10%; therefore, genomic DNA from *A. americanum* ticks was pooled by combining 10 μL from each of 10 ticks. Each pool was tested using 5 μL of the combined template DNA with the appropriate assay (Table 1). Because all ticks comprising a positive pool would be retested individually, a threshold value of the mean background fluorescence plus 10 standard deviations (SDs) was established and the reaction was allowed to run to 55 cycles to increase sensitivity. For each positive pool, DNA from the individual ticks was tested using the parameters described above for the Echaf and Blone qPCR assays of individual ticks.

Assessment of tick genomic DNA for PCR inhibitors

To verify that tick genomic DNA did not contain materials that would interfere with qPCR assays, a dilution series of plasmid DNA that was spiked with tick genomic DNA taken from field-caught adult and nymph *A. americanum* that had tested negative with the Rambl and Rick17 qPCR assays was tested. All probe and primer sets (Table 1) had been tested previously using plasmid-amplified DNA and showed an amplification sensitivity of 3–10 copies (Jiang et al. 2009). Three dilution series of 10–10^7 copies per μL of target *R. amblyommii* plasmid DNA were evaluated by qPCR using the reaction program described above (Table 1). Three replicates of each dilution series were completed using a 25 μL reaction volume containing 4 μL of template, consisting of 2 μL of each dilution of plasmid DNA to which was added either 2 μL of sterile water or 2 μL of tick genomic DNA. Linear regression analysis (PROC REG of SAS® ver. 9.1; SAS Institute, Cary, NC) was used to determine the relationship between the cycle threshold (Ct) number and the concentration of plasmid DNA in each dilution series.

Confirmatory sequencing and phylogenetic analyses

Rickettsia- and *Borrelia*-positive tick samples and three-step thermocycling programs were used with primers listed in Table 2 to produce amplicons for sequencing. Genomic DNA from *A. americanum* that tested positive in Rambl, Rmont, and Blone qPCR assays were selected randomly for sequencing from the total pool of positive ticks, as were *D. variabilis* testing positive for Rick17 only. However *A. americanum* that tested positive for Rick17 only were selected randomly for sequencing within each site. Attempts were made to amplify and sequence *B. burgdorferi* from all positive *I. scapularis*. Amplification products were purified with the QIAquick PCR purification kit (Qiagen) to remove primers and short oligonucleotides. Samples testing positive in species-specific (Rambl, Rmont, Blone, Bburg) or genus-specific (Rick17) qPCR assays were processed separately for sequencing in a laminar flow hood. Sequencing was carried out at the North Carolina State University Genomic Sciences Laboratory using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) or at the Naval Medical Research Center, using a 3100 gene analyzer (Applied Biosystems) with the ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) using the forward primers from Table 2. Bacterial species were identified by the GenBank databases, with the standard nucleotide–nucleotide Basic Local Alignment Research Tool program to ascertain their closest relatives (Altschul et al. 1990). Chromatograms were viewed and edited using 4Peaks software ver. 1.7.2 (http://mekentosj.com/4peaks/).

TABLE 1. PRIMERS AND PROBES USED IN QUANTITATIVE POLYMERASE CHAIN REACTION ASSAYS TO IDENTIFY BACTERIAL PATHOGENS IN TICKS COLLECTED FROM 26 SITES IN CHATHAM COUNTY, NORTH CAROLINA, FROM APRIL TO JULY 2006

Assay *Bacterial species* *Reference*	*Primer/probe*[a] *sequences*	*Target gene (amplicon size)*	*Reaction mixture*[b]
Rick17	R17k135F ATGAATAAACAAGGKACNGGHACAC	17 kDa	F Primer 0.3 μM
Rickettsia spp.	R17k249R AAGTAATGCRCCTACACCTACTC	(114 bp)	R Primer 0.3 μM
Jiang et al. (2004)	R17k202P FAMCCGAATTGAGAACCAAGTAATGCTAMRA		Probe 0.4 μM
Rambl	Ra477F GGTGCTGCGGCTTCTACATTAG	*ompB*	F Primer 0.5 μM
Rickettsia amblyommii	Ra618R CTGAAACTTGAATAAATCCATTAGTAACAT	(141 bp)	R Primer 0.5 μM
Jiang et al. (2009)	Ra532PFAMCGCGATCTCCTCTTACACTTGGACAGAATGCTTATCGCGBHQ1		Probe 0.5 μM
Rrick	RR1370F ATAACCCAAGACTCAAACTTTGGTA	*ompB*	F Primer 0.4 μM
Rickettsia rickettsii	RR1494R GCAGTGTTACCGGGATTGCT	(124 bp)	R Primer 0.4 μM
Jiang et al. (2005c)	RR1425BROXCGCGATCTTAAAGTTCCTAATGCTATAACCCTTACCGATCGCGDAB		Probe 0.4 μM
Rmont	RMF2832 GCGGTGGTGTTCCTAATACRM	*ompB*	F Primer 0.5 μM
Rickettsia montanensis	R2937 CCTAAGTTGTTATAGTCTGTAGTG	(105 bp)	R Primer 0.5 μM
Rozmajzl et al. (2006)	RMB2875FAMCGGGGCAAAGATGCTAGCGCTTCACAGTTACCCCGBHQ1		Probe 0.5 μM
Echaf	ECH16S17F GCGGCAAGCCTAACACATG	16S rRNA	F Primer 0.2 μM
Ehrlichia chaffeensis	ECH16S97R CCCGTCTGCCACTAACAATTATT	(80 bp)	R Primer 0.2 μM
Loftis et al. (2003)	ECH16S38PFAMAGTCGAACGGACAATTGCTTATAACCTTTTGGTBHQ1		Probe 0.2 μM $MgCl_2$ 4.0 mM
Blone	Bl594F TGGTGGAGAAGGTGTTCAAG	*Fla*	F Primer 0.5 μM
Borrelia lonestari	Bl719R GCATTAGCATCAATAGCAGTTG	(125 bp)	R Primer 0.5 μM
Unpublished procedure of the NMRC	Bl655H HEXCGCGACCAGCTCCAGCTCAAGGTGGGATTAGTCGCGBHQ1		Probe 0.3 μM $MgCl_2$ 6.0 mM
Bburg	Bb23Sf CGAGTCTTAAAAGGGCGATTTAGT	23S rRNA	F Primer 0.7 μM
Borrelia burgdorferi sensu lato	Bb23Sr GCTTCAGCCTGGCCATAAATAG	(75 bp)	R Primer 0.7 μM
Courtney et al. (2004)	Bb23SP FAMAGATGTGGTAGACCCGAAGCCGAGTGTAMRA		Probe 0.175 μM

[a]FAM, 6-carboxyfluorcein; HEX, 2′,4′, 5′,7′,1,4-hexachlorofluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine; BHQ1, Black Hole Quencher 1; ROX, tetrapropano-6-carboxyrhodamine; DABCYL, 4-([4′-(dimethyl-amino)-phenyl]-azo) benzoic acid.

[b]Unless listed, dNTP = 0.2 μM, $MgCl_2$ = 5.0 mM, and Taq = 0.75 U.

NMRC, Naval Medical Research Center.

TABLE 2. POLYMERASE CHAIN REACTION PRIMER SETS AND THERMOCYCLER PARAMETERS USED FOR POLYMERASE CHAIN REACTION SEQUENCING REACTIONS IN THE IDENTIFICATION OF PATHOGENS DETECTED IN TICKS COLLECTED FROM 26 SITES IN CHATHAM COUNTY, NORTH CAROLINA, FROM APRIL TO JULY 2006

Gene target	*Primers (source)*	*Reference*	*Thermocycling program*
Rickettsia sp. *ompB* Outside	11F ACCATAGTAGCMAGTTTTGCAG 2553R GAATTTTCAAAAGCAATYGTATCAGT (Sigma, Genosys, The Woodlands, TX)	Jiang et al. (2005a)	94°C 3 min, 3 temp 40 cycles [94°C 30 s, 55°C 30 s, 68°C 3 min], 72°C 7 min
Rickettsia sp. *ompB* Inside	607F AATATCGCTGACGGTCAAGGT 1452R SGTTAACTTKACCGYTTATAACTGT (Sigma)	Jiang et al. (2005a)	94°C 3 min, 3 temp 40 cycles [94°C 30 s, 55°C 30 s, 68°C 1 min], 72°C 7 min
E. chaffensis 16S gene	HE1 CAATTGCTTATAACCTTTTGGTTATAAAT HE3 ATAGGGAAGATAATGACGGTACCTATA (Sigma)	Anderson et al. (1992)	94°C 3 min, 3 temp 40 cycles [94°C 60 s, 55°C 60 s, 72°C 60 s], 72°C 7 min
Borrelia fla gene Outside	11F ATCATAATACGTCAGCTATAAATGC 914R ATACATATTGAGGCACTTGATTTG (Eurofins MWG Operon)	NMRC, unpublished	94°C 3 min, 3 temp 40 cycles [94°C 30 s, 54°C 30 s, 68°C 90 s], 72°C 7 min
Borrelia fla gene Inside	11F ATCATAATACGTCAGCTATAAATGC 551R GCTTCATCCTGATTTGCACCAAC (Eurofins MWG Operon)	NMRC, unpublished	94°C 3 min, 3 temp 40 cycles [94°C 30 s, 54°C 30 s, 68°C 60 s], 72°C 7 min
B. burgdorferi rrf-rrl spacer	ITSF ATTACCCGTATCTTTGGC ITSR TCAATAAATGTTTGCTTCTC (Sigma)	Postic et al. (1998)	94°C 3 min, 3 temp 40 cycles [94°C 60 s, 50°C 60 s, 72°C 60 s], 72°C 7 min

ompB, outer membrane protein B.

An outer membrane protein B gene (*ompB*) fragment was amplified with primer pairs 11F and 2553R followed by a nested amplification using *ompB* primer pairs 607F and 1452R (Table 2). The resulting amplicon was sequenced to identify rickettsial species from *A. americanum*, *D. variabilis*, and *I. scapularis* that tested positive with the genus-specific Rick17 or Rambl qPCR assay. A sequence homology of ≥97% was required for identification of the molecular isolate to species (Stackebrandt and Goebel 1994).

Phylogenetic analysis

A phylogenetic analyses was carried out to determine the relatedness of 28 partial sequences of *ompB* amplified from genomic DNA from ticks that were qPCR positive to Rick17 or Rambl and 11 *ompB* reference sequences obtained from GenBank (*R. montanensis* AF123716, *Rickettsia raoultii* DQ365798, *Rickettsia rhipicephali* AF123719, *Rickettsia massiliae* AF123714, *R. amblyommii* EU728827, FJ455415, *Rickettsia honei* AF123724, *Rickettsia hoogstraalii* EF629536.1, *Rickettsia felis* AF 182279, *R. rickettsii* AY751299, and an *I. scapularis* endosymbiont EF433951). Alignment of *ompB* sequences encompassed ~273 nucleotide positions. Analyses of sequence data were performed with the software package MEGA 4 (Tamura et al. 2007) after multiple alignments by CLUSTAL X (Thompson et al. 1997). The evolutionary distances were computed using the Jukes–Cantor method (Jukes and Cantor 1969), and clustering was performed by the Neighbor-Joining method (Saitou and Nei 1987). A stable tree topology was achieved after a bootstrap algorithm of 1000 repetitions (Felsenstein 1985).

Analysis of spatial distribution pattern of Rickettsia*-infected lone star ticks*

Rates of *Rickettsia* infection were compared among lone star tick populations using replicated G-tests (http://udel.edu/~mcdonald/statrepgtestgof.html). Our hypothesis was that the prevalence of infection of lone star ticks with *Rickettsia* spp. was the same among the populations sampled. An infection rate at each collection site was calculated as a percentage based on the numbers of ticks testing positive for *Rickettsia* (Rick17 or Rambl) relative to the total number of ticks tested. Replicated G-tests (Sokal and Rohlf 1995) were performed using the prevalence of infection over all sites as the expected ratio of *Rickettsia*-positive to *Rickettsia*-negative ticks.

A visual inspection of the prevalence data for rickettsial infections in lone star ticks suggested that there was an inverse relationship between the spatial distribution of ticks infected with *R. amblyommii* and ticks infected with other species of *Rickettsia*. This relationship was evaluated by regression analysis. The prevalence of lone star ticks infected with *R. amblyommii* (Rambl positive) was regressed against the prevalence of other *Rickettsia* spp. (Rick17 positive, but Rambl negative) across all collection sites using the PROC REG procedure of SAS®.

Results

Occurrence and abundance of tick species

A total of 3737 ticks were collected at 26 sites (Fig. 1) from April to July 2006. Of this total, 3695 (98.9%) were *A. americanum* with 342 (9.3%) adult males, 260 (7.0%) adult females, and 3093 (83.7%) nymphs collected. In contrast, only 36 (1%) *D. variabilis* adults (15 males and 21 females) and 15 (0.4%) *I. scapularis* nymphs were collected. Additionally, 4 (0.1%) *I. scapularis* adults were collected in November 2006. *A. americanum* were collected at all sampling sites, including three sites that were public forest lands with no residential development. Low numbers of ticks ($n = 65$) were recovered from the forest sites despite sampling on two separate occasions. Approximately one-third of all adult *A. americanum* were collected at a single site in the northwest portion of the county.

D. variabilis and *I. scapularis* were collected at 7 (26.9%) and 6 (23.1%) of the 26 sites, respectively. However, only two sites yielded more than one *I. scapularis*. Sites where *D. variabilis* were collected yielded an average (±SD) of 3.5 ticks (±3.7) with a single site yielding 12 adults.

Extraction and amplification of genomic DNA

With a protocol and reagents that were largely nonproprietary, genomic DNA was extracted from *A. americanum* that was equivalent or higher in concentration and quality than genomic DNA extracted concurrently with the Qiagen DNeasy kit (Smith MP, Ponnusamy L, Apperson CS. unpublished data). qPCR assays were carried out using a dilution series of *R. amblyommii* plasmid DNA that was spiked with tick genomic DNA from *A. americanum* nymphs or adults, or sterile water. When plotted against known log10 dilution series at each concentration of template, the Ct values for the three dilution series were highly concordant, indicating that tick genomic DNA did not contain inhibitors that would interfere with the qPCR assays. Parameters for regression of each log-transformed dilution series against Ct values were highly significant ($p < 0.001$), and r^2 values for the three regression analyses were all >0.94. Mean intercept (±standard error of mean) and slope (±standard error of mean) values for the regression analyses of plasmid DNA spiked with water, tick nymph DNA, or tick adult DNA ranged from 30.73 (±1.058) to 31.50 (±1.213) and −0.704 (±0.0349) to −0.721 (±0.0396), respectively. When the Ct values of the positive controls were compared between cycler runs, qPCR assays provided highly consistent results with low variance of mean Ct values. Mean Ct values (±SD) for plasmid DNA (2000 copies) as positive controls in Rambl and Rick17 qPCR assays were 32.9 (±1.9, $n = 47$) and 32.3 (±1.5, $n = 14$) cycles, respectively.

Pathogen prevalence

Occurrence of *A. americanum* ticks infected with pathogens is presented in Tables 3 (over all sites) and 4 (by site). *R. amblyommii*, an SFG rickettsia, was detected at all sites with infection prevalence averaging (±SD) 56.4% (±11.1%) and ranging from 35.9% to 76.9%. *A. americanum* that contained unidentified *Rickettsia* spp. occurred at 24 (92.3%) of 26 sites, in an average of 11.8% (±7.2%) of the ticks tested and ranged in prevalence from 4.4% to 30.2%. Over all sites, 68.2% (±9.2% = SD) of *A. americanum* ticks tested positive for *Rickettsia* with infection prevalence ranging from 53.3% to 85.9%. The prevalence of *Rickettsia* in lone star ticks varied significantly among sampled populations in Chatham County (heterogeneity $G = 81.26$; $df = 25.24$; $p < 0.01$). A visual examination (Fig. 2) of the spatial distribution of *A. americanum* infected with *Rickettsia* suggested that an inverse relationship existed between the prevalence of ticks infected with *R. amblyommii* (Rambl positive) and the prevalence of ticks infected with other *Rickettsia* spp. (Rick17 positive and Rambl negative). A regression analysis (Fig. 3) of the percentage of lone star ticks infected with *R. amblyommii* against those infected with other *Rickettsia* spp. verified the negative association, and the slope of the regression line was highly significant ($p = 0.001$).

Testing of 159 pools of genomic DNA derived from 1590 *A. americanum* resulted in 23 (14.4%) pools positive for *E. chaffeensis*. When the individual ticks comprising positive pools were tested, *E. chaffeensis* was detected in 25 (1.6%) *A. americanum* collected from 12 (46.2%) of 26 sites. Infection rates ranged from 1.0% to 15.4% and averaged 1.8% (±3.5%) of ticks tested at all sites and 4.4% (±4.4%) at sites solely where ticks positive for the agent were collected (Fig. 4). Interestingly, 18 (72.0%) of 25 *E. chaffeensis*–positive ticks were coinfected with *Rickettsia*, with 15 (60%) infected with *R. amblyommii* and 3 (12%) with an unidentified species of *Rickettsia* that tested positive with the Rick17 genus-specific qPCR assay (Table 5).

B. lonestari infection was detected in 6 (3.8%) of 159 pools and in 6 (0.4%) of 1590 lone star ticks. Three of the six infected ticks were collected at a single site. Three of the *A. americanum* were coinfected with a *Rickettsia* sp. but no *B. lonestari–E. chaffeensis* coinfections were detected (Table 5).

Compared to *A. americanum*, only low numbers of *D. variabilis* could be tested because few were collected (Table 6). Sixteen (44.4%) of 36 *D. variabilis* tested positive with the genus-specific Rick17, but none of these molecular isolates were found to be positive when subsequently evaluated with

TABLE 3. RESULTS OF QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSES FOR PATHOGENS IN *AMBLYOMMA AMERICANUM* TICKS COLLECTED AT 26 SITES IN CHATHAM COUNTY, NORTH CAROLINA, IN SPRING 2006

	Number testing positive (% testing positive)				
Bacterial species (qPCR assay)	*Male, n = 305*	*Female, n = 245*	*Adults, n = 550*	*Nymphs, n = 1040*	*Overall, n = 1590*
R. amblyommi (Rambl+)	176 (57.7)	155 (63.3)	331 (60.2)	540 (51.9)	871 (54.8)
Rickettsia spp. (Rambl − / Rick17+)	39 (12.8)	33 (13.5)	72 (13.1)	133 (12.3)	205 (12.9)
Total *Rickettsia* (Rambl + and Rick17+)	215 (70.5)	188 (76.7)	403 (73.3)	673 (64.7)	1076 (67.7)
E. chaffeensis (Echaf)	7 (2.3)	8 (3.3)	15 (2.7)	10 (0.96)	25 (1.6)
B. lonestari (Blone)	2 (0.7%)	0 (0.0%)	2 (0.4)	4 (0.4)	6 (0.4)

qPCR, quantitative polymerase chain reaction.

TABLE 4. PREVALENCE OF *AMBLYOMMA AMERICANUM* TICKS INFECTED WITH BACTERIAL PATHOGENS AT 26 SITES IN CHATHAM COUNTY, NORTH CAROLINA, IN 2006

		Range of % positive (mean % positive ± SD)	
Bacterial species (qPCR assay)	*No. sites positive (% positive)*	*All 26 sites*	*Sites where detected*
R. amblyommii (Rambl+)	26 (100)	35.9–72.9 (56.4 ± 11.1)	35.9–72.9 (56.4 ± 11.1)
Rickettsia spp. (Rambl − /Rick17+)	24 (92.3)	0.0–30.2 (11.8 ± 7.2)	4.4–30.2 (12.7 ± 6.5)
Total *Rickettsia* spp. (Rambl + and Rick17+)	26 (100)	53.3–85.9 (68.2 ± 9.2)	53.3–85.9 (68.2 ± 9.2)
E. chaffeensis (Echaf)	12 (46.2)	0.0–15.4 (1.8 ± 3.5)	1.0–15.4 (4.3 ± 4.4)
B. lonestari (Blone)	4 (15.4)	0.0–2.3 (0.2 ± 0.6)	0.7–2.3 (1.5 ± 0.7)

SD, standard deviation.

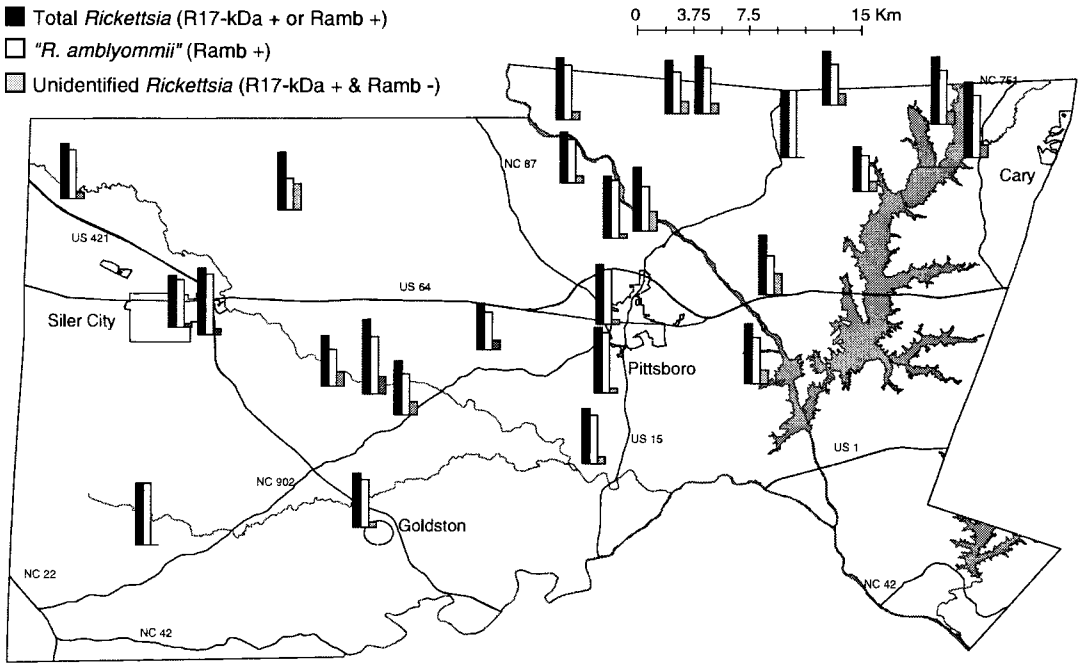

FIG. 2. Percentage of *Amblyomma americanum* testing positive for *Rickettsia* species by qPCR (Rambl and/or Rick17 qPCR assays). Ticks were collected at 26 sites in Chatham County, North Carolina, in the spring of 2006. qPCR, quantitative polymerase chain reaction.

an *R. rickettsii*–specific qPCR assay. The overall prevalence of rickettsial agents identified in *D. variabilis* was 19.4% (7/36) positive for *R. montanensis*, 11.1% (4/36) positive for *R. amblyommii*, and 13.9% (5/36) that were positive only with Rick17 and did not react to any of the species-specific assays used.

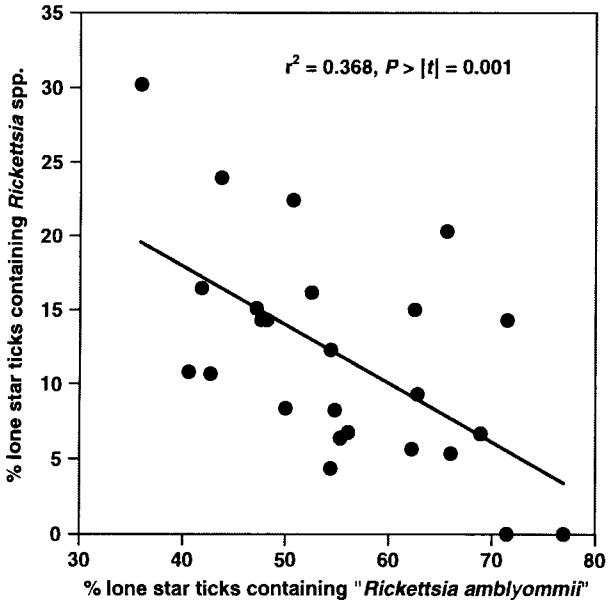

FIG. 3. Regression of percentage of *A. americanum* ticks infected with *Rickettsia amblyommii* and unidentified *Rickettsia* at 26 collection sites in Chatham County, North Carolina.

Similar to *D. variabilis*, because of their low abundance, few *I. scapularis* were tested. Five (33.3%) of 15 nymphs and 1 (25%) of 4 of adults reacted positively to Rick17, but were not tested with *Rickettsia* species–specific qPCR assays. Significantly, 6 (40%) of 15 nymphs collected at 3 out of 6 sites where *I. scapularis* was recovered were positive for *Borrelia burgdorferi* sensu lato, but no adult tested positive.

Identification of molecular isolates

Species identities of some bacterial agents detected by qPCR were confirmed by sequence analyses using nested primers targeting the *ompB* gene. Of nine randomly selected ticks positive in Ramb qPCR assays, seven molecular isolates from *A. americanum* and two from *D. variabilis* were found to be 99–100% homologous to an *R. amblyommii* sequence in GenBank (EU728827). *OmpB* amplicons from seven Rmont-positive *D. variabilis* were 100% homologous to an *R. montanensis ompB* sequence deposited in GenBank (AF123716). Eight molecular isolates from *A. americanum* that were Rambl negative but Rick17 positive yielded useable sequences. One molecular isolate was 99% homologous to *R. amblyommii* (EU728827), six were 99% homologous to *R. montanensis* (AF123716), and one was 99% homologous to *R. honei* (AF123724).

The *ompB* gene fragments from four Rick17-positive *I. scapularis* nymphs were sequenced, with three isolates 100% homologous to *R. massiliae* (DQ503428) and *Rickettsia* sp. Bar29 (AF123710), and the fourth molecular isolate was 96% homologous to *R. felis* (CP000053, AF182279, AF210695). This last isolate may be a novel species as the *ompB* partial sequence was not more than 97% homologous to any rickettsial species in GenBank.

From sequencing reactions, all six isolates of *B. lonestari* from *A. americanum* were successfully sequenced in the *fla*

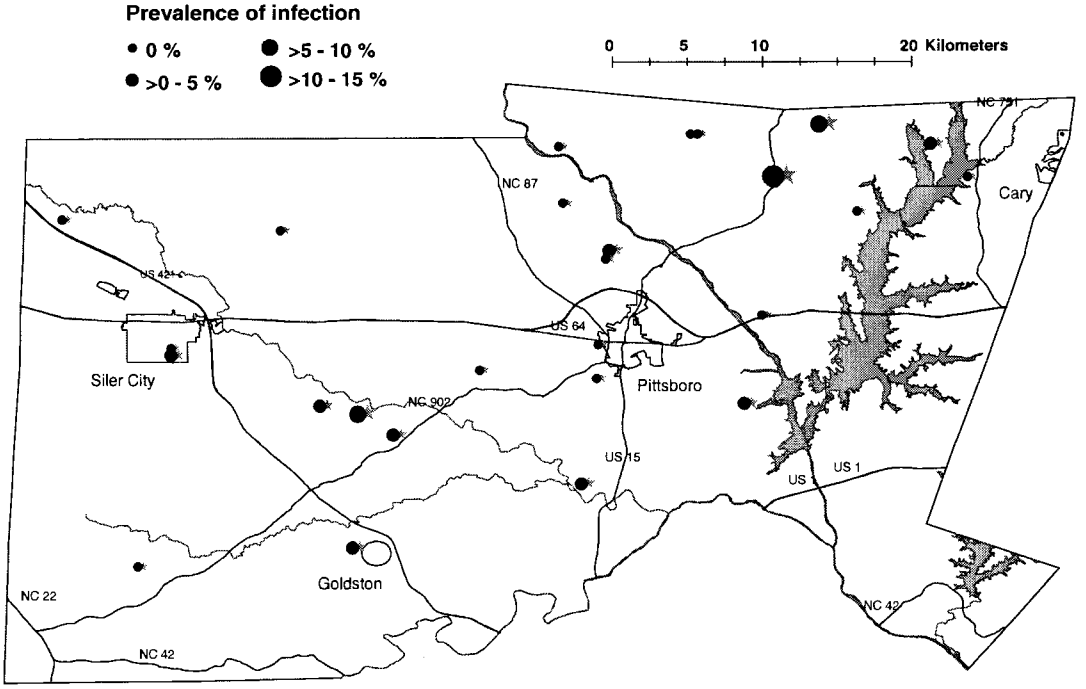

FIG. 4. Percentage of *A. americanum* ticks testing positive by qPCR for *Ehrlichia chaffeensis*. Ticks were collected at 26 sites in Chatham County, North Carolina, from April to July 2006.

(Flagellin) region and showed 99% homology to a GenBank sequence for this species (AY850064). However, only one of six qPCR-positive *I. scapularis* nymphs yielded an amplicon that showed 99% homology to *B. burgdorferi* sensu stricto (AE000783). Although amplicons of the appropriate size were visually detected on 1.0% agarose gels with ethidium bromide staining after PCR amplification of genomic DNA from *E. chaffeensis*–positive ticks, efforts to sequence 16S rDNA gene fragments failed because of the presence of ambiguous sequences, suggesting that ticks may have been infected with more than one *Ehrlichia* species.

Phylogenetic analyses of rickettsiae

Phylogenetic analyses were performed based on the partial sequence of the *ompB* gene of molecular isolates from 28 ticks (Fig. 5). Bootstrap analyses were carried out, and bootstrap values of 50% or greater are shown at the appropriate nodes. As indicated by the extent of sequence similarity, *ompB* gene sequences for 13 of the 28 newly sequenced ticks (*D. variabilis* and *A. americanum*) were placed in the clade of the *R. montanensis* strain M/5-6 (AF123716), which was supported by a bootstrap percentage of 91% in the tree. Three sequences from *I. scapularis* clustered within the *R. massiliae* with a bootstrap percentage of 60%. Eight sequences from *A. americanum* and two sequences from *D. variabilis* placed within the *R. amblyommii* clade. Finally, one sequence from *I. scapularis* placed within the *R. felis* clade.

Discussion

Occurrence and abundance of tick species

In peri-residential landscapes, the predominant tick was *A. americanum* with significantly fewer *D. variabilis* and *I. scapularis* ticks collected. Nymphs comprised 82.8% (3093) of the

TABLE 5. *AMBLYOMMA AMERICANUM* TICKS COINFECTED WITH BACTERIAL PATHOGENS

	Number coinfected/number tested (% coinfected)			
Bacterial species + coinfecting agent (qPCR assay)	*Males*	*Females*	*Nymphs*	*Total*
E. chaffeensis				
+ *R. amblyommii* (Rambl+)	4/7 (57.1)	6/8 (85.7)	5/10 (50.0)	15/25 (60.0)
+ *Rickettsia* spp. (Rambl− /Rick17+)	0/7 (0.0)	1/8 (12.5)	2/10 (20.0)	3/25 (12.0)
+ All *Rickettsia* spp. (Rambl+ and Rick17+)	4/7 (57.1)	7/8 (87.5)	7/10 (70.0)	18/25 (72.0)
Not coinfected	3/7 (42.9)	1/8 (12.5)	3/10 (30.0)	7/25 (23.0)
B. lonestari				
+ *R. amblyommii* (Rambl+)	1/1 (100)	0/0	1/5 (20.0)	2/6 (33.3)
+ *Rickettsia* spp. (Rambl−/Rick17+)	0/1 (0.0)	0/0	1/5 (20.0)	1/6 (16.7)
+ Total *Rickettsia* spp. (Rambl+ and Rick 17+)	1/1 (100)	0/0	2/5 (40.0)	3/6 (50.0)
Not coinfected	0/1 (0.0)	0/0	3/5 (60.0)	3/6 (50.0)

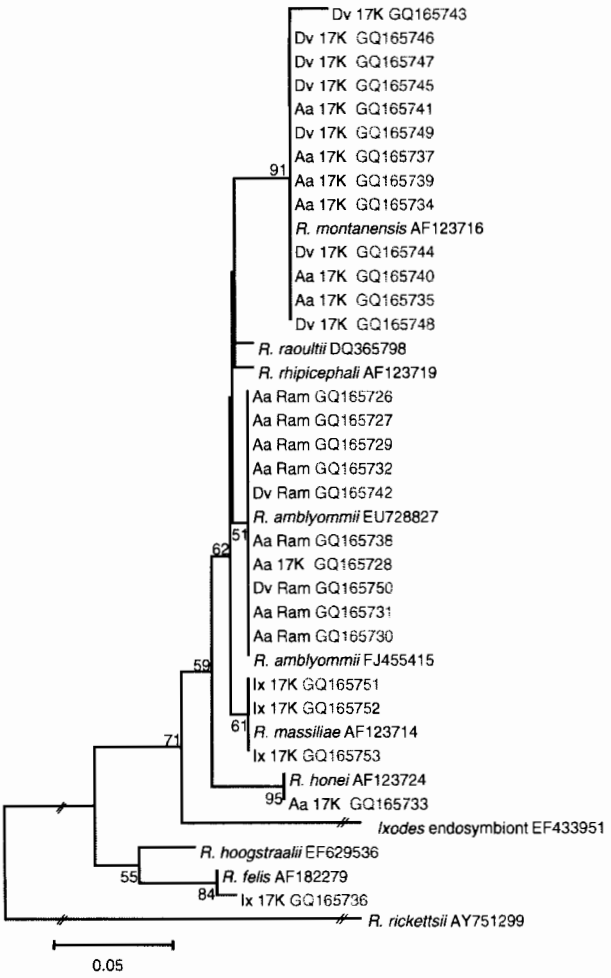

FIG. 5. Neighbor-joining tree showing the phylogenetic relationship of *ompB* partial sequences of *Rickettsia* amplified by PCR from genomic DNA of field-collected *A. americanum*, *Dermacentor variabilis*, and *Ixodes scapularis* to 11 known species of *Rickettsia*. The scale bar indicates an estimated change of 5%. *ompB*, outer membrane protein B.

3695 *A. americanum* collected. Similar results were reported by Apperson and coworkers (2008) for a survey of tick species around residences in Chatham County in 2005. *A. americanum* comprised 99.6% of 6508 ticks collected at 32 separate residential sites, with nymphs accounting for 82.9% of the *A. americanum* collected. Nymphs were seasonally active from April to July, which coincides with the spring–summer period when human activity out of doors would be expected to be high. Taken together, these results indicate that residents in Chatham County would experience highest attack rates from nymphal lone star ticks. The aggressive human-biting behavior and/or high abundance of *A. americanum* relative to other tick species has been previously reported (Ginsberg et al. 1991, Armstrong et al. 2001, Childs and Paddock 2003, Diuk-Wasser et al. 2006, Schulze et al. 2006).

Pathogen prevalence

Several known or potentially pathogenic organisms were detected in the tick population in Chatham County. SFG rickettsiae and, in particular, *R. amblyommii* were widely distributed in the lone star tick populations sampled. In fact, *R. amblyommii* was detected in ticks collected at all 26 residential sites, and over all collection sites, in 54.8% of 1590 *A. americanum* that were individually tested. Apperson et al. (2008) found *R. amblyommii* to be the most prevalent bacterial agent in ticks, occurring in 11 (44%) of 25 pools comprised of 308 lone star ticks and at 7 (38.8%) of 18 residences where ticks were collected. It is likely that *R. amblyommii* was not detected in some ticks at all sampling sites and that estimated infection rates were higher because of the increased sensitivity provided by qPCR assays relative to the conventional PCR assays used by Apperson et al. (2008). *R. amblyommii* appears to be a commonly occurring bacterial agent in lone star ticks. In a survey of nine states, Mixson et al. (2006) reported *R. amblyommii* infection rates to average 41.2%. However, in North Carolina, infection rates for six separate lone star tick populations averaged 56.1%. Clay et al. (2008) conducted a survey of microbial organisms in lone star tick populations from six states, and *R. amblyommii* was detected in every lone star tick population that was tested, ranging in prevalence from 45% to 61% with an estimated 55% of the ticks from one collection site

TABLE 6. RESULTS OF QUANTITATIVE POLYMERASE CHAIN REACTION ANALYSES OF *IXODES SCAPULARIS* AND *DERMACENTOR VARIABILIS* FOR BACTERIAL PATHOGENS

	Number positive/number tested (% positive)				
	Ixodes scapularis		Dermacentor variabilis		
Bacterial agent (qPCR assay)	*Nymphs*	*Adults*	*Males*	*Females*	*Total*
Rickettsia spp. (Rick17)	5/15 (33.3)	1/4 (25.0)	4/12 (33.3)	12/24 (50.0)	16/36 (44.4)
R. amblyommii (Rambl)	NT	NT	1/12 (8.3)	3/24 (12.5)	4/36 (11.1)
R. rickettsii (Rrick)	NT	NT	0/12 (0.0)	0/24 (0.0)	0/36 (0.0)
R. montanensis (Rmont)	NT	NT	2/12 (16.7)	5/24 (20.8)	7/36 (19.4)
B. burgdorferi sensu lato (Bburg)	6/15 (40.0)	0/4 (0.0)	NT	NT	NT

NT, not tested.

in Chatham County, North Carolina, testing positive for this rickettsial organism. Jiang et al. (2009) tested lone star ticks removed from patients reporting to Department of Defense health clinics in 14 states and from scouts and adult leaders attending a Boy Scouts of America camp in Virginia. *R. amblyommii* was detected by qPCR in 58 (80.5%) of 72 lone star tick pools comprised of 340 specimens. In the same study (Jiang et al. 2009), prevalence of *R. amblyommii* was reported to be 69.9% (128/183 ticks) and 63.0% (116/184 ticks) for individual lone star ticks tested in 2002 and 2003, respectively. These reports are reasonably congruent with results of the present investigation, an intensive investigation on a county-wide scale. Consequently, these findings should be generalizable to other areas of North Carolina and the southern United States.

Rickettsiae other than *R. amblyommii* were detected in all three species of ticks that were collected. Through *ompB* sequencing, rickettsial organisms were identified that were highly homologous to *R. montanensis*, *R. honei*, and *R. massiliae*. To our knowledge, detection of *R. montanensis* in *A. americanum* has not been previously reported. Species identifications of other SFG rickettsiae in these ticks would require amplification and sequencing of DNA from other genes. *R. montanensis* appears to be a common microbe associated with *D. variabilis* and has been detected previously in this tick species from Maryland (Ammermann et al. 2004) and from Canada (Dergousoff et al. 2009). *R. rhipicephali* was detected in *D. variabilis* collected in California (Wikso et al. 2008). Notably, *D. variabilis* infected with *R. amblyommii* were not reported in these investigations.

In the present survey, in general, lower prevalence of *E. chaffeensis* (1.6%) was found compared to past investigations. Paddock and Yabsley (2007) reported previously published rates of *E. chaffeensis* infection in *A. americanum* to range between 5% and 15%. In their nine state survey, Mixson et al. (2006) reported *E. chaffeensis* infections averaged 4.7%, but ranged between 0% and 27%. Clay and coworkers (2008) reported the prevalence of *E. chaffeensis*–positive lone star ticks to be 2–3% in the populations sampled from six states. Notably, *B. lonestari* was detected in a smaller percentage (0.4%) of lone star ticks tested. In general, prevalence of infection reported in other surveys has been comparably higher, but only ranging up to 10% (Burkot et al. 2001, Clark 2004, Bacon et al. 2005, Taft et al. 2005, Schulze et al. 2006).

The low prevalence of *E. chaffeensis* and *B. lonestari* in lone star ticks is surprising in view of the strong predilection of lone star ticks to parasitize white-tailed deer (Childs and Paddock 2003, Paddock and Yabsley 2007) and the established role of deer as reservoir hosts of these agents (Paddock and Yabsley 2007). Lively et al. (2005) hypothesized that vertically transmitted pathogens, which are usually mutualists, could limit the prevalence of pathogenic organisms that are primarily transmitted horizontally. Effects of *R. amblyommii*, as a vertically transmitted organism (Clay et al. 2008, Stromdahl et al. 2008), in blocking infection of ticks by pathogenic horizontally transmitted bacterial agents, such as *E. chaffeensis*, are an intriguing prospect. However, larger numbers of *E. chaffeensis*–positive lone star ticks were found to be coinfected with a species of *Rickettsia*, especially *R. amblyommii*, than were found to not be infected with *Rickettsia*. The reverse would be expected if *R. amblyommii* was blocking *E. chaffeensis* infection in lone star ticks. Effects of *R. amblyommii* on tick fitness and molting survival have not been studied, but any negative effects coupled with less than 100% transovarial transmission would be expected to drive the bacteria out of the population unless it is being re-introduced from a horizontal source. In this regard, Stromdahl et al. (2008) found that *R. amblyommii* is transmitted transovarially at less than 100% efficiency, which suggests that *R. amblyommii* may be acquired horizontally. It is well established that white-tailed deer are the principal blood meal host for lone star ticks (Paddock and Yabsley 2007). Notably, Apperson et al. (2008) reported finding only 1 (3%) of 32 deer to be positive for antibodies against *R. amblyommii*. In contrast, 28 (88%) deer were seropositive for *E. chaffeensis* antibodies. These results suggest that lone star ticks are being infected with *R. amblyommii* by feeding on a secondary host. The increase in prevalence of *R. amblyommii* in lone star adults compared to nymphs supports this hypothesis. *R. amblyommii* and *R. montanensis* were detected in both lone star and American dog ticks, which suggests that these ticks were infected from a common host other than deer or that pathogen transmission through cofeeding is involved. Studies involving identification of blood meal hosts of questing lone star and American dog ticks infected with these SFG rickettsiae would be helpful in answering this question.

Collection of *B. burgdorferi* sensu lato–positive *I. scapularis* nymphs is potentially of public health significance in North Carolina. Minimally, these findings indicate that enzootic transmission of *B. burgdorferi* occurs at sites where infected ticks were collected. Lyme disease has been documented in Chatham County (Pegram et al. 1983), and *B. burgdorferi* was cultured from a white-footed mouse (Kirkland et al. 1997) at the site where we collected an infected nymph. However, *I. scapularis* were difficult to collect by flagging, which suggests that this tick species is present in woodlands at low population levels, occupies cryptic habitats that were not sampled, that its questing behavior makes it less vulnerable to collection by flagging. Diuk-Wasser et al. (2006) and Apperson et al. (2008) also collected few *I. scapularis* compared to the large numbers of lone star ticks that were flagged from vegetation. Certainly, additional eco-epidemiology studies of *I. scapularis* are warranted, including efforts to collect and test *I. scapularis* for *B. burgdorferi* in Chatham and other Piedmont counties in North Carolina.

Spatial distribution of A. americanum *infected with* rickettsiae

In the present investigation, the prevalence of *Rickettsia* spp. in lone star ticks was spatially heterogeneous among the populations that were sampled. Clay et al. (2008) reported similar results for lone star ticks sampled from eight different states. Among lone star tick populations in the present investigation, an inverse relationship was found between the prevalence of lone star ticks that were Rambl positive and Rick17 positive/Rambl negative. We realize that this relationship is subject to question, because we did not determine whether *R. amblyommii*–positive ticks were infected with other *Rickettsia* spp. However, if there were no fitness effects from infection or competitive interaction between rickettsial species, then a random spatial distribution of ticks infected with *R. amblyommii* and other *Rickettsia* spp. would be expected. The observed spatial distribution of rickettsiae-infected lone star ticks could be explained by infection

exclusion or interference. Lone star ticks transovarially infected with *R. amblyommii* would potentially be protected from being horizontally infected with other species of *Rickettsia*, resulting in an increase in prevalence of *R. amblyommii*–infected ticks relative to ticks infected with other *Rickettsia*. Infection exclusion by the symbiont *Rickettsia peacockii* has been postulated to explain the decline in *Dermacentor andersoni* females infected with *R. rickettsii* in the Bitter Root Valley of Montana (Kurtti et al. 2005). Infection exclusion (interference) of rickettsial organisms has also been reported for *D. variabilis* (Macaluso et al. 2002, de la Fuente et al. 2003). An understanding of the biotic and abiotic factors that are driving the prevalence of lone star ticks infected with *R. amblyommii* and other *Rickettsia* spp. in opposite directions will require additional research.

Identification of molecular isolates

In general, sequencing of *ompB* verified the species identifications of most *Rickettsia* that were detected by species-specific qPCR assays. Why some lone star ticks that tested negative with Rambl and positive with the Rick17 qPCR were highly homologous to *R. amblyommii* when *ompB* amplicons were sequenced is not known. Genetic variants of *R. amblyommii* may exist that are not detected with the qPCR assay used. In this regard, Papin et al. (2004) produced molecular variants of West Nile virus that were not detected with a commonly used TaqMan assay through point mutations in the target zone of the TaqMan probe binding site. In retrospect, sequencing of Rambl and Rick17 qPCR amplicons may have been informative. Genetic variation in other species of *Rickettsia*, such as *R. rickettsii*, has been reported (Karpathy et al. 2007, Ellison et al. 2008).

Public health implications

Human-biting ticks containing a diverse microbial fauna inhabit peri-residential habitats of Chatham County. Clearly, however, the greatest risk of infection from a tick-borne agent is from SFG rickettsiae transmitted through the bite of lone star ticks. Transmission of these microbes to wildlife hosts may ultimately contribute to new lines of infected ticks (Azad and Beard 1998) that once established pose threats to public health. Further studies focusing on detecting species of *Rickettsia* that infrequently occur in ticks could potentially identify emerging pathogens. In this regard, a single *A. americanum* was found positive for a *Rickettsia* that had 100% homology to *R. honei ompB*. *R. honei* is known to be a human pathogen in Australia and Thailand (Jiang et al. 2005b). Attempts to amplify additional gene targets (*ompA* and *sca4*) for this molecular isolate were unsuccessful. Notably, a similar unconfirmed molecular isolate was detected in *Amblyomma cajennense* collected from cattle in south Texas (Billings et al. 1998). In addition, in the present study, in three *I. scapularis* nymphs, *ompB* sequences similar to *R. massiliae*, a suspected human pathogen in Sicily Italy (Vitale et al. 2006) associated with *Rhipicephalus sanguineus* (Eremeeva et al. 2006), were detected. Although more evidence, such as the sequencing of additional gene targets, would be needed to confirm that these seemingly novel rickettsiae are indeed known pathogens or just analogs, their detection in North Carolina ixodid tick populations suggests that undiagnosed febrile illnesses associated with tick bites could be due to new emerging rickettsial pathogens.

Based on the spatial distribution and prevalence of infected ticks, human exposure to a tick-borne organism would be greatest to *R. amblyommii* transmitted through the bite of lone star ticks. In view of its high prevalence in lone star ticks, widespread geographic distribution and past investigations implicating it as a potential human pathogen (Billeter et al. 2007, Apperson et al. 2008, Stromdahl et al. 2008, Jiang et al. 2009), the eco-epidemiology of *R. amblyommii* merits further research. Presently, beyond its geographic prevalence and vertical transmission in lone star ticks, not much is known about the horizontal transmission or acquisition of this rickettsial agent.

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References

Altschul, SF, Gish, W, Miller, W, Myers, EW, et al. Basic local alignment search tool. J Mol Biol 1990; 215:403–410.

Ammermann, NC, Swanson, KI, Anderson, JM, Schwartz, TR, et al. Spotted-fever group *Rickettsia* in *Dermacentor variabilis*, Maryland. Emerg Infect Dis 2004; 10:1478–1481.

Anderson, BE, Sims, KG, Olson, JG, Childs, JE, et al. *Amblyomma americanum*: a potential vector of human ehrlichiosis. Am J Trop Med Hyg 1993; 49:239–244.

Anderson, BE, Sumner, JW, Dawson, JE, Tzianabos, T, et al. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. J Clin Microbiol 1992; 30:775–780.

Apperson, CS, Engber, B, Nicholson, WN, Mead, DG, et al. Rickettsial disease in North Carolina: Is "*Rickettsia amblyommii*" a possible cause of rickettsiosis reported as Rocky Mountain spotted fever? Vector Borne Zoonot Dis 2008; 8:597–606.

Apperson, CS, Levine, JF, Evans, TL, Braswell, A, et al. Relative utilization of reptiles and rodents as hosts by immature *Ixodes scapularis* (Acari: Ixodidae) in the coastal plain of North Carolina. Exp Appl Acarol 1993; 17:719–731.

Armstrong, PM, Brunet, LR, Spielman, A, Telford, SR, III. Risk of Lyme disease: perceptions of residents of a lone star tick-infested community. Bull WHO 2001; 79:916–925.

Azad, AF, Beard, CB. Rickettsial pathogens and their arthropod vectors. Emerg Infect Dis 1998; 4:179–186.

Bacon, RM, Pilgard, MA, Johnson, BJB, Piesman, J, et al. Rapid detection methods and prevalence estimation for *Borrelia lonestari glpQ* in *Amblyomma americanum* (Acari: Ixodidae) pools of unequal size. Vector Borne Zoonot Dis 2005; 5:146–156.

Billeter, SA, Blanton, HL, Little, SE, Levy, MG, et al. Detection of "*Rickettsia amblyommii*" in association with a tick bite rash. Vector Borne Zoonot Dis 2007; 7:607–610.

Billings, AN, Yu, XJ, Teel, FD, Walker, DH. Detection of a spotted fever group rickettsia in *Amblyomma cajennense* (Acari: Ixodidae) in south Texas. J Med Entomol 1998; 35:474–478.

Burkot, TR, Mullen, GR, Anderson, R. *Borrelia lonestari* DNA in adult *Amblyomma americanum* ticks, Alabama. Emerg Infect Dis 2001; 7:471–473.

Childs, JE, Paddock, CD. The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States. Annu Rev Entomol 2003; 48:307–337.

Clark, K. *Borrelia* species in host-seeking ticks and small mammals in northern Florida. J Clin Microbiol 2004; 42:5076–5086.

Clay, K, Klyachko, N, Grindle, D, Civitello, D, et al. Microbial communities and interactions in the lone star tick, *Amblyomma americanum*. Mol Ecol 2008; 17:4371–4381.

Courtney, JW, Kostelnik, LM, Zeidner, NS, Massung, RF. Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J Clin Microbiol 2004; 42:3164–3168.

de la Fuente, J, Blouin, EF, Kocan, KM. Infection exclusion of the rickettsial pathogen *Anaplasma marginale* in the tick vector *Dermacentor variabilis*. Clin Diagn Lab Immunol 2003; 10:182–184.

Dergousoff, SJ, Gajadhar, AJA, Chilton, NB. Prevalence of *Rickettsia* species in Canadian populations of *Dermacentor andersoni* and *D. variabilis*. Appl Environ Microbiol 2009; 75:1786–1789.

Diuk-Wasser, MA, Gatewood, AG, Cortinas, MR, Yaremych-Hamer, S, et al. Spatiotemporal patterns of host-seeking *Ixodes scapularis* nymphs (Acari: Ixodidae) in the United States. J Med Entomol 2006; 43:166–176.

Duncan, AW, Correa, MT, Levine, JF, Breitschwerdt, EB. The dog as a sentinel for human infection: prevalence of *Borrelia burgdorferi* C6 antibodies in dogs from southeastern and mid-Atlantic states. Vector Borne Zoonot Dis 2004; 4:221–229.

Durden, LA, Oliver, JH, Jr., Banks, GW, Vogel, GN. Parasitism of lizards by immature stages of the blacklegged tick, *Ixodes scapularis* (Acari, Ixodidae). Exp Appl Acarol 2002; 26:257–266.

Ellison, DE, Clark, TR, Sturdevant, DE, Virtaneva, K, et al. Genomic comparison of virulent *Rickettsia rickettsii* Sheila Smith and avirulent *Rickettsia rickettsii* Iowa. Infect Immun 2008; 76:542–550.

Eremeeva, ME, Bosserman, EA, Demma, LJ, Zambrano, L, et al. Isolation and identification of *Rickettsia massiliae* from *Rhipicephalus sanguineus* ticks collected in Arizona. Appl Environ Microbiol 2006; 72:5569–5577.

Felsenstein, J. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 1985; 39:783–791.

Felz, MW, Durden, LA. Attachment sites of four tick species (Acari: Ixodidae) parasitizing humans in Georgia and South Carolina. J Med Entomol 1999; 36:361–364.

Felz, MW, Durden, LA, Oliver, JH, Jr. Ticks parasitizing humans in Georgia and South Carolina. J Parasitol 1996; 82:505–508.

Ginsberg, HS, Ewing, CP, O'Connell, AF, Jr., Bosler, EM, et al. Increased population densities of *Amblyomma americanum* (Acari: Ixodidae) on Long Island, New York. J Parasitol 1991; 77:493–495.

Greene, RT, Levine, JF, Breitschwerdt, EB, Berkhoff, HA. Antibodies to *Borrelia burgdorferi* in dogs in North Carolina. Am J Vet Res 1988; 49:473–476.

James, AM, Liveris, D, Wormser, GP, Schwartz, I, et al. *Borrelia lonestari* infection after a bite by an *Amblyomma americanum* tick. J Infect Dis 2001; 183:1810–1814.

Jiang, J, Blair, PJ, Felices, V, Moron, C, et al. Phylogenetic analysis of a novel molecular isolate of spotted fever group rickettsiae from northern Peru: *Candidatus Rickettsia andeanae*. Ann NY Acad Sci 2005a; 1063:337–342.

Jiang, J, Blair, PJ, Olson, JG, Stromdahl, E, et al. Development of a duplex quantitative real-time PCR assay for the detection of tick-borne spotted fever group rickettsiae and *Rickettsia rickettsii*. Int Rev Armed Forces Med Serv 2005c; 78:174–179.

Jiang, J, Chan, TC, Temenak, JJ, Dasch, GA, et al. Development of a quantitative real-time polymerase chain reaction assay specific for *Orientia tsutsugamushi*. Am J Trop Med Hyg 2004; 70:351–356.

Jiang, J, Sangkasuwan, V, Kriangkrai, L, Sukwit, S, et al. Human infection with *Rickettsia honei* in Thailand. Emerg Infect Dis 2005b; 11:1475–1477.

Jiang, J, Yarina, T, Miller, MK, Stromdahl, EY, et al. Molecular detection of *Rickettsia amblyommii* in *Amblyomma americanum* parasitizing humans. Vector Borne Zoonot Dis 2009 DOI: 10.1089/vbz.2009.0061.

Jukes, TH, Cantor, CR. Evolution of protein molecules. In: Munro, HN, ed. *Mammalian Protein Metabolism*. New York: Academic Press, 1969:21–132.

Karpathy, SE, Dasch, GA, Eremeeva, ME. Molecular typing of isolates of *Rickettsia rickettsii* by use of DNA sequencing of variable intergenic regions. J Clin Microbiol 2007; 45:2545–2553.

Kirkland, KB, Klimko, TB, Meriweather, RA, Schriefer, M, et al. Erythema-like rash illness at a camp in North Carolina—a new tick-borne disease? Arch Int Med 1997; 157:2635–2641.

Kishore, R, Hardy, WR, Anderson, VJ, Sanchez, NS, et al. Optimization of DNA extracted from low-yield and degraded samples using the BioRobot® EZ1 and BioRobot® M48. J Forensic Sci 2006; 51:1055–1061.

Kurtti, TJ, Simser, JA, Baldridge, JD, Palmer, AT, et al. Factors influencing *in vitro* infectivity and growth of *Rickettsia peacockii* (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Acari, Ixodidae). J Invertebr Pathol 2005; 90:177–186.

Levine, JF, Wilson, ML, Spielman, A. Mice as reservoirs of the Lyme disease spirochete. Am J Trop Med Hyg 1985; 34:55–60.

Lively, CM, Clay, K, Wade, WJ, Fuqua, C. Competitive coexistence of vertically and horizontally transmitted parasites. Evol Ecol Res 2005; 7:1183–1190.

Loftis, AD, Massung, RF, Levin, ML. Quantitative real-time PCR assay for detection of *Ehrlichia chaffeensis*. J Clin Microbiol 2003; 41:3870–3872.

Macaluso, KR, Sonenshine, DE, Ceraul, SM, Azad, AF. Rickettsial infection in *Dermacentor variabilis* (Acari: Ixodidae) inhibits transovarial transmission of a second *Rickettsia*. J Med Entomol 2002; 39:809–813.

McQuiston, JH, Paddock, CD, Holman, RC, Childs, JE. The human ehrlichioses in the United States. Emerg Infect Dis 1999; 5:635–641.

Mixson, TR, Campbell, SR, Gill, JS, Ginsberg, HS, et al. Prevalence of *Ehrlichia, Borrelia*, and *Rickettsial* agents in *Amblyomma americanum* (Acari: Ixodidae) collected from nine states. J Med Entomol 2006; 43:1261–1268.

Moore, VA, Varela, AS, Yabsley, MJ, Davidson, WR, et al. Detection of *Borrelia lonestari*, putative agent of southern tick-associated rash illness, in white-tailed deer (*Odocoileus virginianus*) from the southeastern United States. J Clin Microbiol 2003; 41:424–427.

Moyer, PA, Varela, AS, Luttrell, MP, Moore, VA, et al. White-tailed deer develop spirochetemia following experimental infection with *Borrelia lonestari*. Vet Microbiol 2006; 115:229–236.

Oliver, JH, Jr., Lin, T, Gao, L, Clark, KL. An enzootic transmission cycle of Lyme borreliosis spirochetes in the southeastern United States. PNAS 2003; 100:11642–11645.

Ouellette, J, Apperson, CS, Howard, P, Evans, TL, et al. Tick-raccoon associations and the potential for Lyme disease transmission in the coast plain of North Carolina. J Wildl Dis 1997; 33:28–39.

Paddock, CD, Yabsley, MJ. Ecological havoc, the rise of white-tailed deer, and emergence of *Amblyomma americanum*-associated zoonoses in the United States. CTMI 2007; 315: 289–324.

Papin, JF, Vahrson, W, Dittmer, DP. SYBR green-based real-time quantitative PCR assay for detection of West Nile virus circumvents false-negative results due to strain variability. J Clin Microbiol 2004; 42:1511–1518.

Parola, P, Paddock, CD, Raoult, D. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin Microbiol Rev 2005; 18:719–756.

Pegram, PS, Jr., Sessler, CN, London, WL, Burgdorfer, W. Lyme disease in North Carolina. South Med J 1983; 76:740–742.

Piesman, J. Transmission of Lyme disease spirochetes (*Borrelia burgdorferi*). Exp Appl Acarol 1989; 7:71–80.

Postic, D, Marti Ras, N, Lane, RS, Hendson, M, et al. Expanded diversity among Californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* Group DN127). J Clin Microbiol 1998; 36:3497–3504.

Rozmajzl, PJ, Flyer, JG, Jiang, J, Stromdahl, EY, et al. A novel qPCR assay used to detect *Rickettsia montanensis* in *Dermacentor variabilis* ticks removed from DoD personnel. 2006 Joint Meeting: The 20th Meeting of the American Society for Rickettsiology and The 5th International Conference on *Bartonella* as Emerging Pathogens Asilomar Conference Grounds, Pacific Grove, CA. September 2–7, 2006.

Ryan, JR, Apperson, CS, Orndorff, PE, Levine, JF. Characterization of Lyme disease spirochetes isolated from ticks and vertebrates in North Carolina. J Wildl Dis 2000; 36:48–55.

Saitou, N, Nei, M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406–425.

Schulze, TL, Jordan, RA, Healy, SP, Roegner, VE, et al. Relative abundance and prevalence of selected *Borrelia* infections in *Ixodes scapularis* and *Amblyomma americanum* (Acari: Ixodidae) from publicly owned lands in Monmouth County, New Jersey. J Med Entomol 2006; 43:1269–1275.

Slaff, M, Newton, NH. Location of tick (Acari: Ixodidae) attachment sites on humans in North Carolina. J Med Entomol 1993; 30:485–488.

Sokal, RR, Rohlf, FJ. *Biometry: The Principles and Practice of Statistics in Biological Research*, 3rd edition. New York: W.H. Freeman, 1995:715–724.

Stackebrandt, E, Goebel, BM. Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994; 44:846–849.

Steiert, JG, Gilfoy, F. Infection rates of *Amblyomma americanum* and *Dermacentor variabilis* by *Ehrlichia chaffeensis* and *Ehrlichia ewingii* in Southwest Missouri. Vector Borne Zoonot Dis 2002; 2:53–60.

Stromdahl, EY, Vince, MA, Billingsley, PM, Dobbs, NA, et al. *Rickettsia amblyommii* infecting *Amblyomma americanum* larvae. Vector Borne Zoonot Dis 2008; 8:15–24.

Taft, SC, Miller, MK, Wright, SM. Distribution of *Borrelia* among ticks collected from eastern states. Vector Borne Zoonot Dis 2005; 5:383–389.

Tamura, K, Dudley, J, Nei, M, Kumar, J. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24:1596–1599.

Thompson, JD, Gibbon, TJ, Plewniak, F, Jeanmougin, F, et al. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25:4876–4882.

Vitale, G, Mansueto, S, Rolain, J, Raoult, D. *Rickettsia massiliae* in human isolation. Emerg Infect Dis 2006; 12:174–175.

Wikso, ME, Hu, R, Dasch, GA, Krueger, L, et al. Detection and identification of spotted fever group rickettsiae in *Dermacentor* species from Southern California. J Med Entomol 2008; 45: 509–516.

Address correspondence to:
Allen L. Richards
Viral and Rickettsial Diseases Department
Naval Medical Research Center
503 Robert Grant Avenue
Silver Spring, MD 20910-7500

E-mail: allen.richards@med.navy.mil

Charles S. Apperson
Department of Entomology
North Carolina State University
Box 7647
Raleigh, NC 27695-7647

E-mail: charles_apperson@ncsu.edu